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Release of Transgenic Hypovirulent Fungi

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Objectives and Specific Aims: RNA viruses of the family *Hypoviridae* reduce virulence of the chestnut blight fungus *Cryphonectria parasitica*. This phenomenon, termed hypovirulence, has effectively reduced the severity of chestnut blight in European chestnut forests and orchards. Cytoplasmic transmission of hypovirus RNA by naturally occurring hypovirulent strains is restricted by a vegetative compatibility (v-c) system that regulates the ability of *C. parasitica* strains to anastamose (fusion of hyphae). The high level of v-c diversity found in native fungal populations is believed to contribute to the reduced level of efficacy of natural hypovirulent strains for biological control of chestnut blight in North American forest ecosystems.

We have modified *C. parasitica* strains to contain a nuclear DNA copy of the prototypic hypovirus genomic RNA. These "transgenic hypovirulent" strains are able to transmit virus to sexual (ascospore) progeny. This novel virus transmission property is predicted to provide increased biological control potential by circumventing barriers imposed by the v-c system. In conjunction with a separate study of biological control efficacy, we are performing a risk assessment analysis of an intense deployment protocol for transgenic hypovirulent *C. parasitica* (Biotechnology permit #96-275-01). The anticipated results will provide a valuable database for risk assessment predictions for future field release of genetically engineered fungi. The specific aims include:

1) Surveying nontargeted woody species for infection by transgenic hypovirulent *C. parasitica*.

2) Surveying insect populations for recovery of transgenic strains.

3) Determining the effect of transgenic strain deployment on *C. parasitica* genetic population structure.

4) Examining the phenotypic variability of recovered transgenic strains.

Results: 1999 was the second year of application of three transgenic *C. parasitica* strains deployed as sprayed asexual spores (conidia) to a clear cut test plot located in the Meshomasic State Forest in central Connecticut. Application dates were May 11, June 3, June 22, July 13, August 3 and August 24, 1999. In each application, the 23.5m by 13m treatment plot

containing 23 chestnut sprout clumps was sprayed with water containing between 10^{11} and 10^{12} spores of a mixture of the transgenic strains. The 31m by 13m control plot, separated from the treatment plot by a 13.3m by 18.3m buffer zone and containing 24 chestnut sprout clumps, was sprayed with water only.

Specific Aim 1: Two to 4 bark samples were collected from cankers or lesions (one canker per tree) found on 11 nontarget woody species (ie., other than American chestnut) surrounding the test plot in an area 15 to 50 meters beyond its perimeter in 1999. Tree species sampled included black birch, white birch, American beech, red oak, chestnut oak, red maple and hickory. Bark samples from these nontarget trees yielded neither natural nor transgenic *C. parasitica* isolates. Thus, there remains no indication that transgenic hypovirulent strains behave differently from natural *C. parasitica* strains in terms of host range specificity. Existing and newly established cankers on nontarget woody species will be sampled and re-sampled at least once per year for the remainder of the project.

Specific Aim 2: Insects have been reported to transmit reproductive propagules (spores, mycelial fragments) of *C. parasitica*, making them potential vectors for introduced transgenic strains. Insects were collected from various parts of the treatment and control plots on June 3, June 22 and July 13, 1999 using a sweep net. Insects collected from the foliage of 6 chestnut sprout clumps in each plot were killed with fumes of 95% ethanol, stored on ice, identified and then cultured on a malt/tannic acid medium that favors growth of *C. parasitica* over other fungi. No *C. parasitica* isolates were recovered from individual insects that included ants, spiders, leafhoppers, grasshoppers, beetles, wasps, gnats and flies, representing 6 taxonomic orders. Further refinements in recovering *C. parasitica* from insect field isolates are in progress.

Specific Aim 3: The long term effect of transgenic strain deployment on the *C. parasitica* genetic population structure is being monitored by characterizing isolates collected from a) cankers on chestnut sprout clumps within the test plot each spring prior to the initial seasonal application of transgenic spores and b) cankers on chestnut trees surrounding the test plot throughout the growing season. Sampling of cankers within the test plot has been intentionally restricted in the early years of this study due to the limited number and small size of cankers. Only five cankers were sampled from within the treatment plot in 1998, while 21 chestnut cankers were sampled from outside the treatment in July of 1998. Not unexpectedly, all of the 26 canker samples taken during the first season yielded nontransgenic *C. parasitica* strains. In 1999, all cankers in the control and treatment plots were sampled as well as cankers on 8 American chestnut trees outside of the plots. *C. parasitica* colonies were recovered from 56 of 62 cankers sampled on 19 of the 23 trees in the control plot, from 45 of the 46 cankers sampled from 18 of the 23 trees in the treatment plot and from all 8 cankers sampled outside of the plots. None of the recovered isolates from either plot were found to be transgenic. However, colonies recovered from cankers on 5 independent trees in the treatment plot were found to contain hypovirus dsRNA. RT/PCR confirmed that these dsRNAs were cytoplasmically transmitted from input transgenic strains to *C. parasitica* field strains.

1999 was also the first year that sexual fruiting bodies (perithecia) were collected for isolation of ascospore progeny. Transgenic ascospore progeny were recovered from cankers on 9 of 13 trees sampled in the treatment plot, while only non-transgenic ascospore progeny were recovered from cankers on 10 trees sampled in the control plot. Thus, there is now good evidence for transmission of cDNA-derived hypovirus RNA independent of the input transgenic strains and of large scale transmission of viral cDNA through mating to ascospore progeny within the treatment plot. However, no evidence has yet been obtained for spread of transgenic strains or derived viral RNA to the control plot or outside of the treatment plot. The recovered *C. parasitica* isolates are being characterized with respect to vegetative compatibility diversity and DNA fingerprint analysis to assess trends in population structure. Control plot isolates have been tentatively placed in 13 v-c groups.

Specific Aim 4: All recovered transgenic ascospore progeny are being analyzed for any signs of phenotypic variability.

Plans for coming year: Dates in 2000 have been scheduled for pre-treatment isolation of *C. parasitica* from cankers in treatment and control plots and on nontarget trees (April), transgenic spore application (4 dates from June through August), insect collection (3 dates from June through August), sampling from chestnut cankers outside of the test plot (September) and collection of sexual fruiting bodies (October). Protocols developed and optimized during the previous year will be employed to meet the goals of each specific aim.